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CHITIN ANALYSIS OF INSECTS (MEALWORM AND CRICKET)

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Tiivistelmä/Referat – Abstract
The literature review dealt with the chemical and physical properties of chitin polymer and its degradation product glucosamine. Particular emphasis was given to studies on the analytical methods of determination of chitin from various products including gravimetrical, spectrophotometric and chromatographic methods.

The aim of this study was to optimize an analytical method to determine insect chitin and to apply the method to quantify chitin in whole insects and their soluble protein fractions. Two species of insects were selected: mealworm larvae (*Tenebrio molitor*) and cricket (*Acheta domesticus*). Whole insects and their protein fractions were treated with alkaline to remove protein. Chitin in de-proteined insect material was then hydrolysed into glucosamine using 6M HCl. Glucosamine from the hydrolysate was determined using chromatographic and spectrophotometric methods, where chromatographic method was optimized and validated.

UPLC-FLR method was specific for glucosamine and the UPLC system could separate two target peaks (glucosamine isomers: α and β). Glucosamine content was calculated using a calibration curve which showed excellent linearity in range 0.0033~24.0 ng/inj. with the determination coefficient more than 0.999 during the study period. The instrumental limit of detection and limit of quantification were 0.00095 ng/inj. (2 µL) and 0.0033 ng/inj. (7 µL), indicating a satisfied sensitivity. Recovery of glucosamine spiked to sample matrix (de-proteined cricket flour) following chitin hydrolysis was not satisfactory (~75%) using HPLC-FLR method, which indicated that spectrophotometric method gave chitin amount closer to the true value due to a higher recovery (>90%). Major findings on chitin amount was ~5% on dry matter basis in both mealworm and cricket. A small percentage of chitin was found in insect protein fractions extracted by 0.1M NaCl.

Avainsanat – Nyckelord – Keywords
Chitin, glucosamine, UPLC, spectrophotometer, mealworm, cricket

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PREFACE

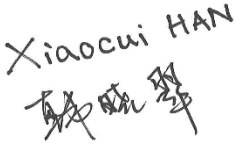
At the beginning of 2017, I had a small talk with Professor Marina Heinonen where I got the concept of chitin and the situation of edible insects as food in Europe. I found this topic highly interesting and fascinating, and it gave me the first push to do my master's thesis on chitin analysis in insects (mealworm and cricket).

This experimental work was carried out at the Department of Food and Environmental Sciences (changed to Department of Food and Nutrition since 2018), Food Chemistry Division, at University of Helsinki in the summer of 2017.

I owe my sincerest thanks to my supervisor, Professor Marina Heinonen, for giving me the opportunity of working in such an interesting topic and supporting my work. Marina, thanks for your patient guidance, advice and encouragement on my lab work and thesis writing. I also warmly thank everyone at EE-building for sharing their knowledge on food chemistry and helping me around the lab.

Further, I would like to thank my fellow students Min Wang, Terhi Lukkari, Mathias Rudolf Amundsen, Rui Wu, Yue Song, Afrizal Afrizal, Maijuleena Salminen, Hongbo Zhao, Tami De Zuani and Airu Song, for accompanying and bringing a lot of memories. Last, but not least, my family, Xiaoyu Han, Shengping Han and Rongfang Shen, deserve a particular note of thanks: all your kind and warm words have, as always, served me well.

Helsinki, January 2018



Xiaocui Han

LIST OF ABBREVIATIONS

ADF	Acid detergent fiber
AQC	6-Aminoquinolyl-N-hydroxy-succinimidyl carbamate
CS	Chitosan
D.M.	Dry matter basis
DR	Derivatizaion reagent
FMOC-Su	N-(9-fluorenylmethoxy-carbonyloxy) succinimide
FLR	Fluorescent detector
GC	Gas chromatography
GlcN	Glucosamine
GlcNAc	N-acetyl-D-glucosamine
HILIC	Hydrophilic interaction chromatography
HPLC	High performance liquid chromatography
IS	Internal standard
MBTH	3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate
NDF	Neutral detergent fiber
OPA	O-phthalaldehyde
PH-HPLC	Normal-phase high-performance liquid chromatography
PITC	Phenylisothiocyanate
RP-HPLC	Reversed-phase high-performance liquid chromatography
R.T.	Room temperature
UPLC	Ultra-high-performance liquid chromatography
UV	Ultraviolet

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1 INTRODUCTION

Insects as food and feed are of increasing interest worldwide, due to the rising cost and demand of animal protein, environmental pressure and population growth (Huis et al. 2013). In Europe, insects as food have low acceptance mainly because they never play a substantial role in food culture and the feelings of disgust (Laureati et al. 2016). Even though, the growing interest of insects as a protein alternative initiate the development of regulations and standards for the use of insects in food and feed. Whole insects and their products are novel foods subject to safety evaluation prior to entering the market (Regulation (EC) No 2015/2283). European Food Safety Authority (EFSA) has assessed the potential risks posed by the use of insects in food and feed (EFSA 2015).

Insect rearing for food and feed could be of great interest for two reasons: (1) insects are an important source of protein and other nutrients; (2) their use as food has ecological and economic benefits over conventional meat in the long run. The nutritional values of edible insects are a highly significant food source for human populations. The crude protein content of insects varies from 13 to 77% of dry matter (D.M.) (Rumpold and Schlüter 2013). The protein quality is considered good because of its high essential amino score (46~96%) and high digestibility (77~98%) (Rumpold and Schlüter 2013). Fat content of insects is of large variance (5~50% crude fat D.M.) (Rumpold and Schlüter 2013). Oils extracted from several insects are rich in polyunsaturated fatty acids and frequently contain the essential linoleic (C18:2) and α-linolenic acids (C18:3) which are important for the healthy development of children and infants (Rumpold and Schlüter 2013). Carbohydrates are mainly represented by chitin derived from insect exoskeleton. Chitin content ranges between 11.6~137.2 mg/kg (D.M.) (Finke 2007). Many species are believed to be good sources of minerals and vitamins. Iron and zinc levels in some insect species are higher than beef (Huis et al. 2013). Their inclusion in daily diet could improve iron/zinc status and help prevent anaemia and zinc deficiency (Huis et al. 2013). Vitamins B (thiamine B1, riboflavin B2, cobalamin B12), retinol (vitamin A) and β-carotene (pre-vitamin A), vitamin E (2R-α-tocopherol) have been detected in some species (Finke 2007; Oonincx and Poel 2011; Tong et al. 2011).

In terms of environmental and economic advantages, insects have a higher feed-conversion efficiency than conventional livestock (cattle, poultry); they can be reared on organic side streams, which reduces environmental contamination; they emit fewer greenhouse gasses and ammonia, which prevents the climate change; rearing insects requires less water and space than cattle, which reduces the cost; they have few animal welfare issues and pose a low risk of transmitting zoonotic infections, etc. (Huis et al. 2013).

Insects can be processed and consumed in three ways: as whole insects, in powder or paste form and as an isolate (e.g. protein, fat/oil, and chitin) (Huis et al. 2013). In Europe, the powdered form of the whole insects, or insect isolation e.g. protein, fat or chitin is relatively accepted compared to the visible whole insects. Insect protein fraction is of the most interest thus it has initiated many related studies, for example, the amino acid profile, thermal stability, solubility, gelling, foaming and emulsifying capacity of insect protein fraction(Yi et al. 2013; Mariod 2013; Zhao et al. 2016; Azagoh et al. 2016; Kim et al. 2016). Besides, insect fat isolate from e.g. melon bug and the sorghum bug, is studied and applied for frying meat and other food products (Mariod et al. 2005 and 2006).

Insect chitin isolate is rarely studied currently, but it is of great commercial potential as a biopolymer and the source of chitin-derived products e.g. chitosan, glucosamine and N-acetyl-D-glucosamine. All of them could be applied in food, medicine, agriculture, bio-nanotechnology and cosmetics. Crustacean-derived chitin has been approved in Japan for use in cereals as a source of fiber and calcium (Belluco et al. 2013); if insect protein concentrates from de-chitined insects became acceptable and were produced on a large scale, the chitin, as a by-product, could be also of great value. Chitin also results in the overestimation of crude protein and decrease the digestibility of protein as well as amino acids. In insect protein research, it seems that when extracting protein from insects, chitin may be also extracted, which may interfere with most protein analysis. The main aim of this study was to optimize analytical method to determine insect chitin and to apply the method to quantify chitin content in whole insects and their protein fractions.

2 LITERATURE REVIEW

2.1 Structure of chitin

Chitin is a linear polymer composed of β (1→4) linked N-acetyl-D-glucosamine (GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose) (Figure 1) (Roberts 1992).

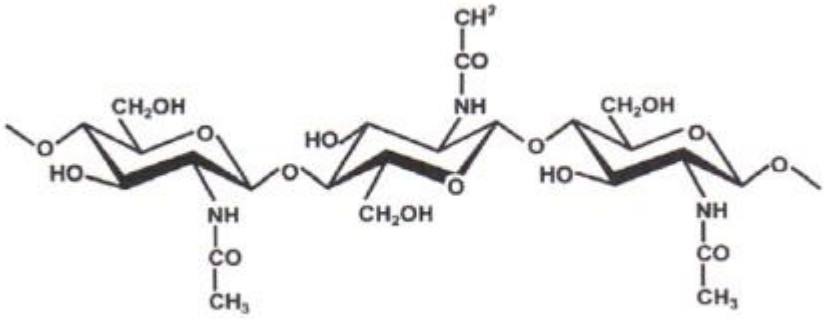


Figure 1. Chemical structure of chitin (Roberts 1992)

GlcNAc, the monomeric unit of chitin, is a derivative of glucose which is the monomer of cellulose. Therefore, chitin is considered as the cellulose derivative with one hydroxyl group on each monomer replaced by an acetyl amine group. Like cellulose, chitin mainly functions as the structural polysaccharides to support the cell and body surfaces: chitin strengthens insect exoskeletons, crustacean shells and fungal cell walls whereas cellulose strengthens the cell wall of plant cells (Gooday 1990). Besides, chitin is also considered as a modified polysaccharide that contains nitrogen, as well as an amide of acetic acid due to the presence of acetylated amino group (Roberts 1992).

Pure chitin (fully N-acetylated) as a homo-polymer is rarely found in nature, since there is always a limited de-acetylation/de-N-acetylation (Roberts 1992). The fraction of N-acetylated units in chitin polymer (F_A) is between 0.9 and 1.0 (Roberts 1992). In this case, chitin may also contain about 5~10% amino groups. The reasons for this slight de-N-acetylation of chitin might be the hydrolysis caused either by the chitin deacetylases in chitin-containing organisms or the chemicals used during the industrial or commercial extraction process (Roberts 1992).

2.2 Occurrence

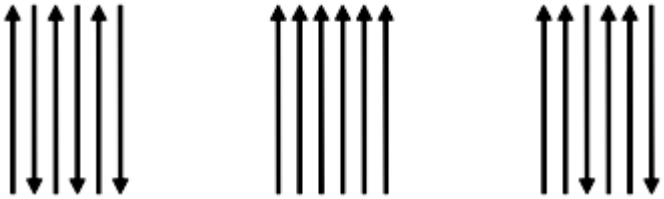
Chitin is the second most widespread natural polysaccharide after cellulose on earth. It can be found in the exoskeleton of all animals with an exo-/outer-skeleton such as crustaceans (e.g. crabs, shrimps, and lobsters) and insects, and in the cell walls of fungi (Nwe et al. 2010).

2.2.1 Biosynthesis

Chitin biosynthesis in living organisms takes place in three steps: in the first step, chitin synthase promotes the polymerization of GlcNAc in the presence of divalent cations (e.g. Mg^{2+}) as co-factors, which forms the polymer chain; in the second step, the native chitin chain is translocated across the membrane and released into the extracellular space; in the third step, the chitin polymer chains are assembled to form crystalline micro-fibrils/nano-crystals (Merzendorfer 2006). Subsequently these nano-crystals cluster into chitin-protein fiber, creating a network whole space are filled with pigments, nano-sized inorganic compounds and other substances (Merzendorfer 2006).

2.2.2 Crystal structure

In nature, chitin is found in crystal structure where the chitin chains form hydrogen-bonded sheets linked by C=O and H-N-groups. Besides, there are intramolecular hydrogen bonds between the neighboring sugar rings in each chitin chain: the carbonyl group bonds to the hydroxyl group on C-6. A second hydrogen bond between the OH-group on C-3 and the ring oxygen also exists, similar to that in cellulose, which adds to the stiffness of the chitin (Minke and Blackwell 1969). Chitin crystalline structure exists in three polymorphic forms: α, β and γ (Hackman and Goldberg 1965) (Figure 2). In α-chitin, the polymer chains are arranged in an anti-parallel orientation and it is considered as the most crystalline and compact form (Carlström 1957); β-chitin consists of parallel chains, while in γ-chain two out of three chains are parallel with the third oriented in the opposite direction (Roberts 1992).



(1) (2) (3)

Figure 2. Arrangement of the chitin polymer chains in three forms
(1) α-chitin, (2) β-chitin, and (3) γ-chitin (Hackman and Goldberg 1965)

Within the crystalline area, the chitin chains are arranged differently. The three chitin forms may have different hydration degree, size and the number of chitin chains per unit cell. These forms may occur in one organism, providing different functional properties: α-chitin is found where extreme hardness is required and it is by far the most abundant form (Rudall and Kenching 1973); β- and γ-chitin have some physiological functions other than supporting since they tend to be tough, flexible and motile (Muzzarelli 1977). α-Chitin has the poorest solubility among the three forms because of the close packing of the chains and its strong inter- and intro-molecular hydrogen bonds (Minke and Blackwell 1969). While β-chitin can swell readily in water and it has a lower chemical and thermal stability because it has relatively weak inter-chain hydrogen bonds (Blackwel 1969).

2.2.3 Cross-linking to other compounds

Because of the availability of reactive free amino groups, chitin chains tend to cross-link to other compounds e.g. calcium carbonate, proteins, lipids, carbohydrates and pigments, and further aggregate to form fibrils in living systems. In animals (both insects and crustaceans), chitin is covalently or non-covalently linked to specific proteins by reaction with α-amino acids (Brine and Austin 1981; Sromova and Lysek 1990). In the cuticle of crustaceans, there are also various degrees of mineralization of chitin associated with calcium carbonate (Hild et al. 2008). Carotenoids occur in many different species of insects and crustacean shells and they are combined with chitin amino groups by carbonyl-amino or Schiff's base-type linkages (Fox 1973). Chitin can also bound to polysaccharides such as mannans, glucans,

galactans, and some other hetero-polysaccharides. In fungal cell walls, glucan chains are linked to chitin through their reducing ends via amino acids, particularly lysine, as well as the hydrogen bond among themselves (Sietsma and Wessels 1981). In all these chitin-combined forms, a certain degree of de-acetylation has been determined, giving a structure between chitin and chitosan.

2.3 Health effects and possible safety concerns

Chitin is considered an indigestible dietary fiber, even though the enzyme chitinase is found in human gastric juices (Paoletti et al. 2007). Unlike the other common dietary fibers of plant origin, chitin is predominantly of animal origin, so it is also called ‘animal fiber’ (Kouřimská and Adámková 2016).

Chitin is found to defend against parasitic infections and some allergic conditions, which improves the immune response of specific groups (Muzzarelli 2010; Huis et al. 2013). However, this immunological response seems to depend on the particle size of chitin: medium and large-sized chitin particles are considered to induce allergic inflammation (Muzzarelli 2010); while small-sized chitin particles may have the reverse effect of reducing the inflammatory response (Brinchmann et al. 2011). People especially those lacking chitinase can have an allergic reaction when eating chitin-containing insects, making chitin an allergen (EFSA 2015). An intake of 5 g of chitin-glucan from crustaceans does not raise a public health concern (EFSA 2010).

The indigestibility of chitin-N can reduce the digestibility of protein (Makkar et al. 2014). Pretorius (2011) reported that the amino acid digestibility of the house fly meal as a broiler diet was over 90% while the digestibility of crude protein was much lower which might be explained by the existence of chitin-N. In insects, the most common form of fiber is chitin which may decrease the insect crude protein digestibility.

2.4 Commercial applications

The major application of chitin is to produce chitin-derived products, such as chitosan (CS), oligosaccharides, glucosamine (GlcN) and N-Acetyl-glucosamine (GlcNAc). Currently the increasing sales of GlcN as a dietary supplement in the market is the driving force for chitin commercial application, about 65% of the chitin produced being converted into GlcN (Sandford 2002).

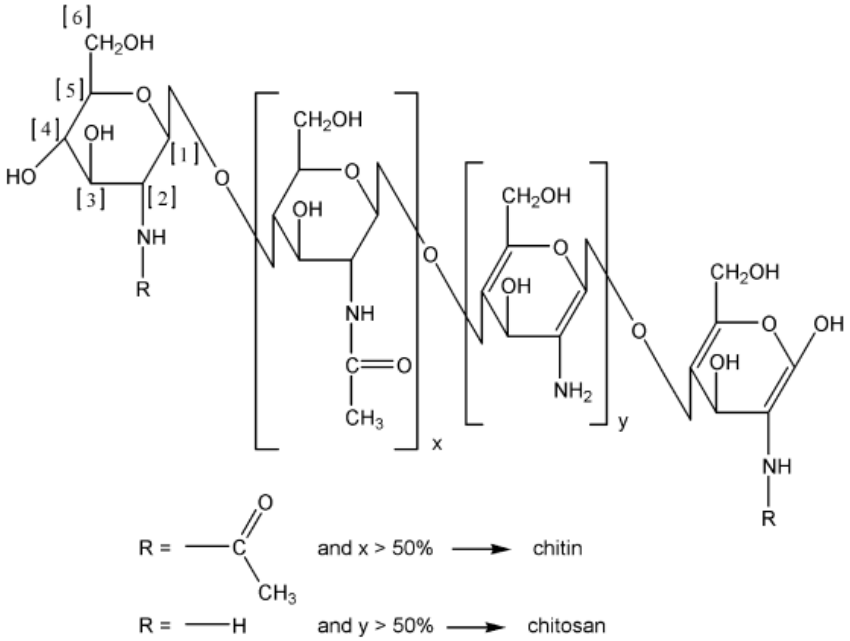
Chitosan (CS, Figure 3) is the partially or fully de-N-acetylated chitin, which is industrially prepared by alkaline de-N-acetylation of chitin (Hirano 1996). It has water-, fat- and dye-binding capacity, as well as emulsifying properties. CS is widely used for water treatment, food preservation, agriculture, cosmetics and medicine (Sandford 2002). CS has also been considered as a potential intelligent and biodegradable polymer for food packaging (Cutter 2006; Portes et al. 2009).

GlcN (Figure 3) is the building block of CS. It is commercially prepared via acid hydrolysis of chitin, involving the cleavage of both the glycosidic and N-acetyl linkages (Novikov and Ivanov 1997). The most popular GlcN product is its dietary supplement in salt forms: chloride and sulphate, both having been clinically proven for treating osteoarthritis with very few side-effects (Anderson et al. 2005). GlcNAc (Figure 3) is a minor commercial product. It is prepared either by chemical acetylation of GlcN using acetic anhydride, or by enzyme catalyzed hydrolysis of chitin (Haynes et al. 1999). GlcNAc is used as a food additive as an inexpensive, less sweet alterative to sucrose for beverages, candies, and instant soup in Japan (MHLW 1995). In human, GlcN, GlcNAc and glucose (Glc) are the main precursors of the disaccharide units in glycosaminoglycans, which are necessary to repair and maintain healthy cartilage and joint function (Mobasheri et al. 2002).

Chito-oligo-saccharides are beta-1, 4 linked homo- or hetero-oligomers of GlcN and/or GlcNAc. Both can be prepared by enzymatic or by chemical de-polymerization of chitin or CS. they have been reported to have anti-microbial, anti-fungal, anti-oxidant, and immunostimulant effects (Kim and Rajapakse 2005). They are water soluble due to their

short chain lengths and free amino groups in GlcN units. The great solubility of chito-oligo-saccharide at neutral pH, together with its low viscosity attracts the interests of many researchers to use chitosan in its oligo-saccharide form.

Besides, chitin polymer itself can be used as a non-absorbable carrier for highly concentrated food ingredients e.g. food dyes and nutrients (Kardas et al. 2012). Microcrystalline chitin (MCC), small polymer of chitin, has good emulsifying, thickening, gelling properties for stabilizing foods. MCC is also used as dietary fiber in baked foods and as a food additive to enhance the flavor and taste of food. For example, as tiny particles, MCC distributes itself evenly throughout aqueous solutions; when heated to normal cooking temperatures, MCC forms pyrazines, which are responsible for the roasted tasted and aroma of several foods (Kardas et al. 2012).



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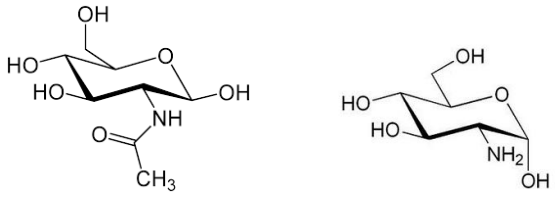


(2) (3)

Figure 3. Structures of (1) chitin and chitosan, (2) GlcNAc, and (3) GlcN (Pillai et al. 2009)

2.5 Industrial extraction of chitin

Chitin in organisms is tightly bound in complexes with other substances, so procedures of chitin isolation are always associated with quantitatively removing of these compounds. Generally, these compounds can be removed by chemical or biological methods. The chemical method requires the use of acids and bases, while the biological method involves microorganisms.

2.5.1 Chemical methods

The traditional method for preparing chitin is a chemical method, generally involving 3 steps: (1) de-mineralization or decalcification, (2) de-proteinization, and (3) de-colorization or bleaching.

Demineralization is done using inorganic or organic acids like HCl, HNO_3, H_2SO_3, CH_3COOH, HCOOH (formic acid), with HCl being the most commonly used reagent at a concentration of 0.25~2M under 0~100 °C for 1~48 h (Kardas et al. 2012). The solid-to-solvent ratio is in the range of 1:10 till 1:40 (w/v) (Kardas et al. 2012). To avoid chitin depolymerization, ethylenediamine-tetra-acetic acid (EDTA) is an alternative used for removal of mineral salts (Kardas et al. 2012). Also room temperature is favored for prevention of chitin de-polymerization (Kardas et al. 2012).

Protein is generally removed via alkaline treatment. Chemicals like potassium hydroxide, sodium carbonate, potassium carbonate, calcium hydroxide, or sodium sulfate at various concentrations in aqueous solutions are applied, with 0.125~2.5M NaOH at 65~100 °C for 1~72 h being commonly used; the solid-to-solvent ratio ranges from 1:10 till 1:20 (w/v) (Kardas et al. 2012).

De-colorization/bleaching can be achieved using oxidants such as $KMnO_4$ with or without H_2O_2, NaClO/HCl, or H_2O_2/HCl. However, when carotenoids and pigments recovery is important for economical account, solvent extraction is used to recover pigments (e.g.

astaxanthin, melanin) (Kardas et al. 2012). Examples of solvents used include acetone, chloroform, ethyl acetate, and ethanol.

However, there is always a certain degree of chitin degradation during these extraction processes, especially where harsh chemical conditions are used (Percot et al. 2003). For example, the use of strong acids/bases leads to chitin de-polymerization and deacetylation, which has detrimental effects on the molecular weight. Besides, the waste liquid may cause environmental pollution. The use of enzymes e.g. proteases, trypsin, chymotrypsin, and papain, is an alternative of strong alkali to remove protein from shells, but there are always a certain amount of protein residues in the chitin isolation and the reaction time is longer than alkaline de-proteinisation (Synowiecki and Al-Khateeb 2000).

2.5.2 Biological methods

Nowadays, microbial demineralization and de-proteination are of high interest. The principle is that bacteria can produce organic acids and protease for de-mineralization and de-proteinisation, separately. This method has been applied in chitin extraction from crustacean waste, for example, by fermentation of shrimp waste with both lactic acid bacteria and non-lactic acid bacteria (Jung et al. 2006; Sini et al. 2007). One advantage of this method is that it allows obtaining a liquid fraction rich in protein, minerals and astaxanthin and a solid chitin fraction (Rao et al. 2000). Besides, organic acids can be produced by bacteria at low cost and the resulting organic salts from the demineralization process are considered environmentally friendly.

2.5.3 Other methods

Qin et al. (2010) also reported a one-step isolation of chitin from shrimps using 1-ethyl-3-methylimidazolium acetate which is an ionic liquid and can not only dissolve chitin but also raw crustacean shells. The advantage is that this liquid is considered green, but with a relatively high cost. Other novel extraction technologies about chitin extraction include microwave and freeze pump-pit thaw cycles.

2.5.4 Identified challenges

There are always impurities left in the chitin fraction. For example, chitin and chitosan from mushrooms were contaminated with glucan or other polysaccharides (Yen and Mau 2007a, b); chitin from insects may contain melanin (Nemtsev et al. 2004). Moreover, high-molecular weight and pure chitin are essential for its use as a raw material to make high quality chitosan. So a great deal of interest still prevails for the optimization of the extraction procedure to minimize the degradation of chitin, meanwhile reducing impurities down to a satisfactory level for specific applications, especially on an industrial scale.

2.6 Quantification of chitin

Quantification of chitin in biological materials or other chitinous products is challenging, since chitin is always associated with other compounds in biological materials. Moreover, chitin is a high molecular polymer and it is insoluble in water and most solvents, so direct quantification of chitin is almost impossible. But indirect quantification which is done by the measurement of its degradation product GlcN, is possible. This is easily performed using two steps: (1) hydrolysis of chitin to produce GlcN, and (2) the measurement of the concentration of GlcN. The major quantitative measurements of GlcN are colorimetric and chromatographic methods. Prior the GlcN analysis, sample pre-treatment procedures, e.g. removing the non-chitin compounds, the degradation/hydrolysis of chitin into GlcN, and sometimes the derivatization of GlcN, are necessary for chitin-contained materials.

2.6.1 Pretreatment

2.6.1.1 Removal of non-chitin compounds

Similarly to chitin extraction, the procedures to remove other compounds such as proteins, carbohydrates, lipids, pigments, minerals that might interfere with the chitin analysis are described in Session 2.5. During this step, the removal of proteins is of the most importance since amino acids (supposed to be from hydrolyzed proteins) could interfere with GlcN analysis, in both colorimetric and chromatographic methods (Ekblad and Näsholm 1996).

2.6.1.2 Degradation/hydrolysis of chitin polymer to yield GlcN

Degradation of chitin polymer to produce GlcN includes two hydrolysis reactions: the cleavage of the β-(1→4) glycosidic bond (de-polymerization) and the removal of the acetyl group (de-N-acetylation). This can be achieved by many mechanisms, e.g. acid-, alkaline- and enzyme-catalyzed hydrolysis, oxidative-reduction free radical de-polymerization, electromagnetic radiation, sonication and mechanical energy (Hai et al. 2003; Einbu and Vårum 2007).

HCl hydrolysis is most studied and is favored among all the methods, because it is effective in both the hydrolysis of glycosidic linkage and acetyl groups, which insures the complete hydrolysis of chitin to GlcN (Hackman 1962; Einbu and Vårum 2007). The concentration of HCl (or pH of the reaction medium), temperature, and heating/incubation time are prime attributes affecting chitin hydrolysis and GlcN recovery, since an excess of acid treatment results in the breakdown of GlcN and further decrease the GlcN recovery (Rupley 1964; Einbu and Vårum 2007). Hackman (1962) found that the degradation of the chitin chain to form oligosaccharides occurred during the first few minutes of the acid hydrolysis, then the produced oligosaccharides undergo further degradation to produce GlcNAc, and finally yield product GlcN and acetic acids. In other words, the de-polymerization of chitin firstly happens and then the deacetylation. The de-polymerization increases strongly with an increased acid concentration, while the deacetylation reaction is only moderately affected by the acid concentration (Einbu and Vårum 2007). The maximum hydrolysis of chitin and GlcN recovery can be obtained under conditions of 6~8 M HCl, 100~110 °C, 4~13 h (Ekblad and Näsholm 1996; Zhu et al. 2005; Crespo et al. 2006).

Alkaline and enzymes are not used as frequently as acids. Base treatment can release CS via de-N-acetylation of chitin polymer, and CS can be further treated to release GlcN for quantification (Ride and Drysdale 1972; Boyle 1995). Enzymatic hydrolysis of chitin is commonly used to release N-acetyl-chito-oligosaccharides with a polymerization between 2 and 5, as well as the monomer unit GlcNAc. This enzymatic treatment is preferred in industrial production of GlcNAc as an orally administrated supplement, due to its specificity

and efficiency. However, it requires more than one type of enzymes to obtain GlcN from chitin, which makes it not favored in quantification of GlcN.

2.6.1.3 Derivatization of GlcN

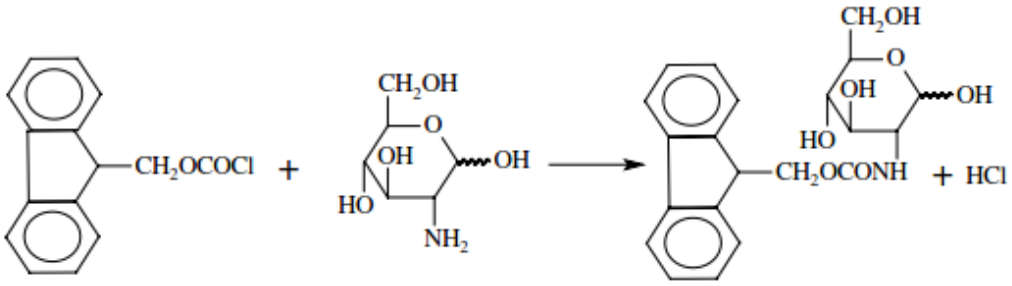
When using a chromatographic method combined with optical detectors, a pre-column derivatization of GlcN is required due to the lack of chromophores and fluorophores by GlcN itself (Díaz et al. 1996). The principle of derivatization is that derivatization reagents (DR) have the groups with chromatophores or fluorescence; then the reaction between the reagent and GlcN produces a GlcN-derivative that can be detected by either ultraviolet (UV) absorption or with fluoresce. Basically, reagents used for amino acids analysis can also derivatize amino sugars including GlcN since the amino group is responsible for the reaction. Previously reported reagents for GlcN derivatization include: 9-fluorenylmethyl chloroformate (FMOC), o-phthalaldehyde (OPA), 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate (AQC) and pHenylisothiocyanate (PITC) (Zhou et al. 2005; Zhu et al. 2005; Harvey 2011).

FMOC, OPA, AQC yield fluorescent derivatives. FMOC is frequently reported to derivatize GlcN, since GlcN-FMOC derivative is stable at room temperature for several hours and shows excellent chromatographic behavior on RP-HPLC (Zhou et al. 2005; Zhu et al. 2005). One disadvantage of FMOC is that it is reactive towards water, forming corresponding alcohol FMOC-OH as a hydrolysis product. FMOC-OH elutes out and appears on the middle of the chromatogram (Díaz et al. 1996). Excess FMOC-OH can overlap with other analytes e.g. amino acids which might be a concern in GlcN analysis (Díaz et al. 1996). FMOC itself also fluorescent, so excess reagent should be removed via extraction with other organic solvents before chromatographic separation. GlcN-OPA derivatives shows low stability within few minutes, but with an online derivatization, the produced GlcN-OPA can be analyzed immediately after derivatization, which avoids the degradation of GlcN-OPA (Díaz et al. 1996; Eikenes et al. 2005). AQC is frequently reported for amino acid and amino sugar derivatization. The derivatization reaction can complete within seconds and the excess of this reagent does not need to be removed (Cohen and Michaud 1993; Díaz et al. 1996; Bosch

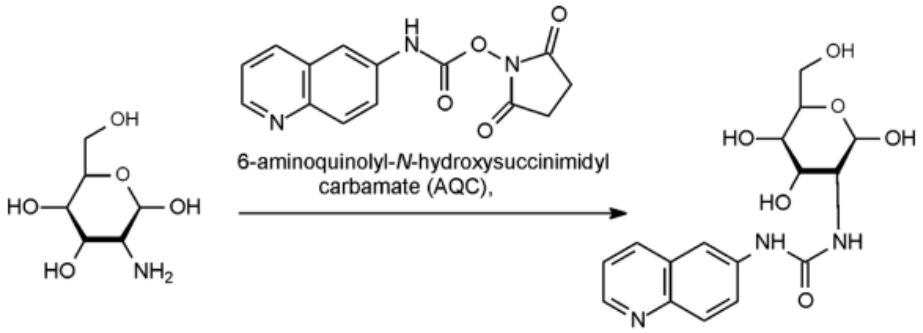
et al. 2006; Wang et al. 2008). GlcN-AQC derivatives are stable for one week and do not decrease in signal until 3 weeks after derivatization (Díaz et al. 1996). PITC yields derivatives detectable by UV. But the derivatization reaction time is long (20 min) and it involves several stages of drying under vacuum to remove the excess reagent (Anumula and Taylor 1991; Hagen 1993; Bosch et al. 2006). All the derivatizing reactions are presented in Figure 4.

2.6.1.4 Internal standard

When the sample preparation procedure includes multiple steps, which can cause the loss of the analytes, internal standard (IS) is added to correct the loss. In the sample pretreatment procedure for GlcN analysis, IS is added prior to the derivatization. The reported IS for GlcN analysis includes L-cysteic acid, L-norleucine, D-galactosamine and α-aminobutyric acid (Hagen 1993; Díaz et al. 1996; Flannery et al. 2001; Li et al. 2013).



(1)



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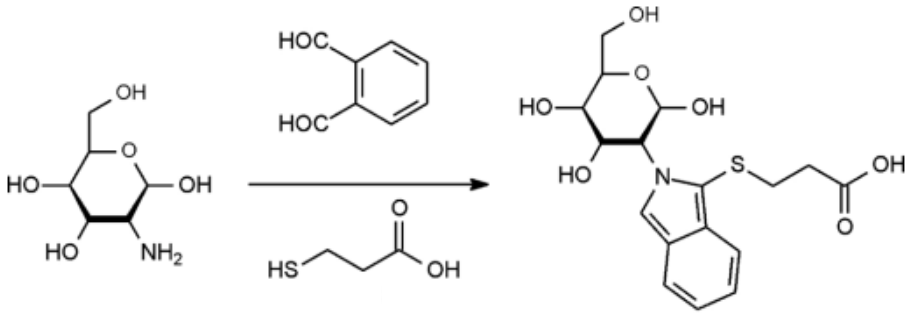
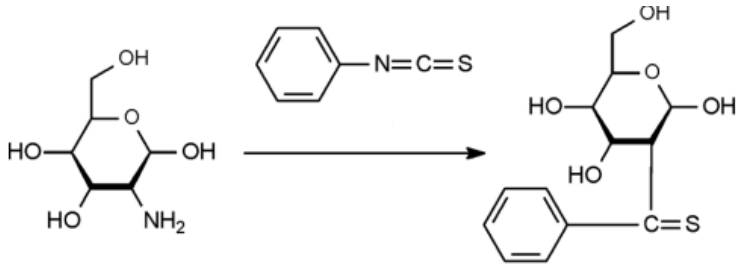


(3)



(4)

Figure 4. Derivatization reactions of GlcN with four different reagents:
(1) FMOC-Cl (2) AQC (3) OPA (4) PITC (Zhu et al. 2005; Harvey 2011)

2.6.2 Measurement of GlcN

2.6.2.1 Colorimetric methods

A frequently used colorimetric assay is the MBTH (aldehyde/3-methyl-2-benzothiazolone hydrazine) method. This method is based on the findings that 2,5-anhydrohexoses (II) produced by nitrious deamination of hexosamine (I) (including fructosamine, galactosamine, glucosamine and mannosamine) react readily with MBTH (III) to exhibite a blue color by addition of a ferric chloride solution. The intense of this color is measured as absorbance spectrophotometrically at wavelength 650/653 nm (Tsuji et al. 1969a, b). The possible mechanism of this color reaction is shown in Figure 5. This method has been used to quantify fungi chitin which can be applied to a wide range of solid substrates including living plant tissue, decaying wood, leaf litter, food products and cereal grains (Ride and Drysdale 1972; Matcham et al. 1984; Frey et al. 1994; Bierstedt et al. 1998; Chen and Chiou 1999). The disadvantage of this method is that it is not specific, since it cannot differentiate between GlcN and other hexosamines such as fructosamine, galactosamine, and mannosamine which might be present in high quantities in some materials (Ekblad and Näsholm 1996).

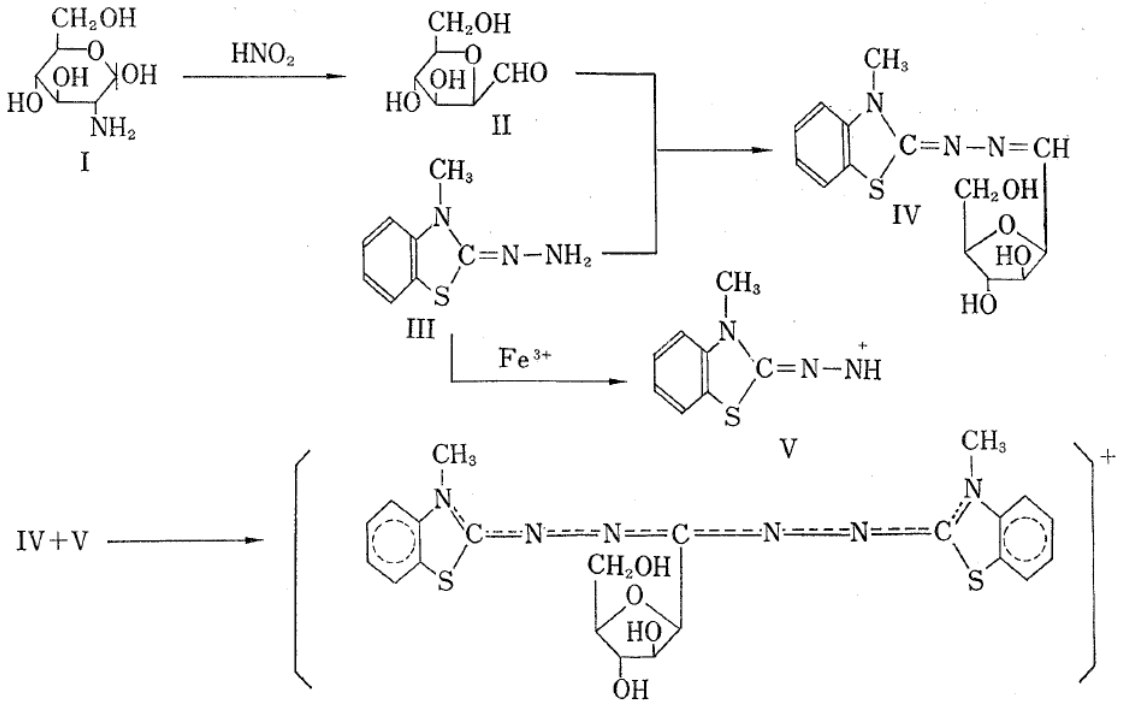


Figure 5. Possible mechanism of the color reaction (Tsuji et al. 1969a,b)

Ninhydrin is another reagent that is able to react with GlcN, producing a purple color compound with a maximum absorbance at 570nm (Wu et al. 2005). However, Ninhydrin also reacts with alpha-amino acids producing a blue-to-purple color compound, which also has the absorbance at detection wavelength. In this case, it interferes with GlcN analysis especially when the sample composition is much more complicated other than pure GlcN tablet/dietary supplements (Wu et al. 2005).

2.6.2.2 Chromatographic methods

Chromatographic methods refer to the separation techniques combining with different spectrometric detection methods. Other detection, such as electrochemical detection can also be used. In the separation step, gas chromatography (GC) and high-performance liquid chromatography (HPLC) are commonly used, with HPLC being preferred in GlcN analysis, since GC always requires sample derivatization to volatiles, which adds to the time and cost.

In HPLC, the separation is based on the analytes' distribution between the stationary phase and mobile phase, which is decided by the polarity differences between the two phases (Fanali et al. 2013). Both reversed-phase (RP) and normal-phase (NP) HPLC are used to separate GlcN or GlcN-derivatives, with RP-HPLC being widely used. In RP-HPLC mode, the polarity of the mobile phase is higher than the stationary phase (Fanali et al. 2013). The frequently used stationary phases are hydrophobic bonded silica particles whose surfaces have been modified with C_{18} or C_8 chains; the combined mobile phases are a mixture of organic solvents (e.g. methanol or acetonitrile) and aqueous solution (pure water, or buffer solution including sodium salts e.g. sodium citrate, sodium acetate, sodium acetate trihydrate etc.), with water being the major percentage, either isocratic or gradient elution (Díaz et al. 1996; Zhu et al. 2005; Eikenes et al. 2005; Zhou et al. 2005; Crespo et al. 2006; Wang et al. 2008; Song et al. 2012; Yan and Evenocheck 2012; Li et al. 2013).

One advantage of RP-HPLC analysis of GlcN is that the equilibration of RP-HPLC columns is fast, which is ideally suited for gradient elution (Fanali et al. 2013). The columns are repeatable and relatively stable provided certain precautions (Fanali et al. 2013). The

predominant mobile phase, water, is inexpensive and plentiful and samples in aqueous solvent can be directly injected. However, GlcN is a highly polar molecule, it is not well restrained by these column packing materials like C18 and C8, with or without ion pairing mobile phases, compared to the columns used in NP-HPLC system (Shao et al. 2004; Roda et al. 2006).

Hydrophilic interaction chromatography (HILIC), one type of NP- HPLC, is also used in GlcN separation (El-Saharty and Bary 2002; Shao et al. 2004; Roda et al. 2006; Wang et al. 2008). In HILIC mode, frequently used stationary phase is made of polar material e.g. amino (NH_2) column, and the combined mobile phase is non-polar ones (Fanali et al. 2013). Normally, the mobile phase is a mixture of organic solvents and water/salt buffer, which is similar to the ones used in RP-HPLC but with a high proportion of organic solvents. In this mode, water acts as the strong solvent to elute out GlcN (Fanali et al. 2013). Compared to RP-HPLC, HILIC has a better retention for GlcN. But the column packing material, e.g. NH2 column, is less stable with a shorter using life than C18 columns (Fanali et al. 2013).

The most commonly used detectors combined with HPLC for GlcN detection are fluorescent (FLR) and ultraviolet (UV) detectors. Other detectors such as refractive index (RI), electrochemical (ECD), mass spectrometry (MS) are also used. Generally, FLR and UV detectors require pre-column derivatization of GlcN (Session 2.6.1.3). In FLR, the GlcN-derivative is excited by shorter wavelength energy and emit higher wavelength radiation which is called fluorescence in the detector. And the radiation/emission is measured at the right angles to the excitation (Fanali et al. 2013). FLR has higher sensitivity, selectivity of GlcN than UV and other detectors. However, the required pre-column derivatization procedure is time-consuming and laborious. Besides, during analysis, there are more instrumental variables to account for during optimization, since changes in fluorescence occur with pH and viscosity. The pre-column derivatization of GlcN is also required in UV detector since GlcN itself has weak UV absorbance. The absorbance of GlcN-derivative under UV wavelength (usually between 190 and 600 nm) can be detected.

RI has been reported for the detection GlcN from dietary supplement, baby formula and crab shells (El-Saharty and Bary 2002; Crespo et al. 2006; Wang et al. 2008). In RI, the refractive index of the analytes changes depending on the light and it is in a proportional amount to the concentration (Fanali et al. 2013). One advantage of RI in GlcN detection is that it does not require pre-column derivatization of GlcN, which saves time and the cost (El-Saharty and Bary 2002; Crespo et al. 2006). But it is non-selective since it registers all substances with different RI than the mobile phase. It is generally less sensitive than FLR and UV detectors. Moreover, RI detector is very sensitive to changes of ambient temperature, pressure and flow rate, so it could not be used for gradient elution (Fanali et al. 2013). MS is useful for both quantification and identification of GlcN in various materials, but the high cost and the skills needed for running MS instruments make it not suitable for the routine analysis of GlcN. Electrochemical detection of GlcN requires extensive sample preparation and clean-up procedures, which makes it difficult for samples in complex matrices (Roda et al. 2006).

2.6.3 Other methods

The easiest way to determine the chitin level in a sample might be a gravimetrical method which is done by weighing chitin that can be extracted. This method is especially suitable for samples like crustacean shells and other samples which contain mainly chitin in addition to proteins and minerals. For example, chitin content could be estimated as the dietary fiber amount, acid detergent fiber (ADF) and neutral detergent fiber (NDF). Both ADF and NDF are gravimetrically determined as the insoluble residue remaining after extraction by an acidified quaternary or neutral detergent liquid (Barker et al. 1998; Finke 2007 and 2013; Sánchez-Muros et al. 2016).

The determination of acetic acid liberated from complete hydrolysis of chitin in HCl is also used to quantify chitin, where chitin is considered as fully N-acetylated (Holan et al. 1980). Non-protein nitrogen, which is calculated as crude protein adjusted by total amino acids, is used to estimate the chitin content in crustacean shells (Díaz-Rojas et al. 2006; Langille et al. 2012). In this case, crude protein is determined using the traditional Kjeldahl method by instrumental methods based on the combustion of the sample to release nitrogen that can be

further detected by thermal conductivity in an appropriate equipment; the analysis of amino acids is done using chromatographic methods.

2.7 Quantification of insect chitin

Recently the chitin content in insects has been considered as acid detergent fiber (ADF). Finke (2007 and 2013) found that there was high amount of amino acids in the ADF fraction from insects. Thus, ADF corrected by total amino acids are used as the chitin content. Chitin content in insects are highly dependent on species variations. Even within the same species, the growth conditions and different growing stages have big effect on their chitin levels.

2.8 Other characteristic analysis of insect chitin

Characterization of insect chitin is also of interest for researchers and various industry fields. The crystal structure, degree of N-acetylation, molecular weight and purity of chitin from some insects has been studied (Sajomsang and Gonil 2010). The chemical structure and physiological property of chitin extracted from 6 insect species most existing in Egypt (including bugs, beetles, cockroaches, wasps, hoppers etc.) are found similar to shrimp chitin and they are suitable for CS production (Badawy and Mohamed 2015).

3 EXPERIMENTAL WORK

3.1 Aims

The aim was to optimize an analytical method to determine insect chitin and to apply the method to quantify chitin in whole insects and their soluble protein fractions. Firstly, alkaline conditions to remove proteins from insects to a level not interfering with GlcN determination was studied. Secondly the hydrolysis of chitin from de-proteined insects to obtain the maximum yield of GlcN using HCl was studied. The third objective was to measure GlcN in insects using UPLC-FLR and spectrophotometric methods, where the UPLC-FLR method was optimized and validated.

3.2 Materials and methods

3.2.1 Materials

Insect samples including mealworm larvae (*Tenebrio molitor*) and crickets (*Acheta domesticus*) in this study were provided by Pohjolan Hyönteistalous Oy and Entocube Oy, respectively. The mealworms used in this study were at larval stage and the crickets at adult stage. A commercial D (+)-Glucosamine hydrochloride supplement (Orion Corporation, Finland) was used to optimize the UPLC-FLR method. Reagents and other chemicals used during the experimental work were listed as below:

- D (+)-Glucosamine hydrochloride (GlcN-HCl), ≥99% pure, Sigma
- Sodium hydroxide(NaOH), solid pellets (Merch, Germany)
- Hydrochloric acid 37% (12M) (HCl) (Merch kgaa, Germany)
- Sodium nitrite ($NaNO_2$) (Merch, Germany)
- Potassium hydrogen sulphate ($KHSO_4$) (Merch, Germany)
- Ammonium amidosulfonate ($NH_4SO_3NH_2$) (Merch kgaa, Germany)
- 3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH), reagent grade 99.0% (HPLC) (Sigma-Aldrich)
- Iron (III) chloride($FeCl_3$) ferric chloride, reagent grade 97% (Sigma-Aldrich)
- N-(9-fluorenylmethoxy-carbonyloxy) succinimide (FMOC-Su), (Sigma-Aldrich)
- Acetonitrile (CH_3CN/ACN), HPLC grade (Sigma-Aldrich)

- Triethylamine (TEA), $N(CH_2CH_3)_3/Et_3N$, minimum 99% pure (Sigma-Aldrich)
- Trifluoroacetic acid (TFA) (CF_3COOH), minimum 99% pure (Sigma-Aldrich)
- Water used throughout the whole laboratory work was purified by the Milli-Q equipment (Millipore Corp., Bedford, MA, USA), LC grade

3.2.2 Drying of freshly frozen insects

Freshly frozen whole insects were stored in -20 ºC in plastic boxes as small portions after purchased. They were first freeze-dried to remove the moisture until constant weight, and then ground into powder and sealed in plastic bags. Insect flours were kept in desiccator under -20 ºC to keep the original composition for later use in chitin analysis.

3.2.3 Removal of protein

Proteins were removed by NaOH solution from whole insect flours and their soluble protein fractions prior to GlcN analysis. Both cricket and mealworm insect flours were treated in the same way to study the effect of incubation time on removal of protein. The freeze-dried insect flour was mixed with a serial NaOH solutions at different concentrations 0.125, 0.2 and 0.5M (0.5 g/10 ml w/v), agitated with a stirrer at room temperature according to time: 0, 2, 4 hrs. After each 2-hour incubation, the mixture was centrifuged (12 000 rpm, 5 °C, 15 min) and the supernatant containing protein was discarded. The pellet was washed with distilled water to remove the added Na^+ and OH^- until neutral. The process was repeated twice to achieve the 4-hour incubation. The pellet was freeze-dried until constant weight. The de-proteined insect flour was stored in desiccator at room temperature for later use. To check whether the proteins were removed to a level not interfering with GlcN detection, the de-proteined insect flour was followed hydrolysis and UPLC-FLR analysis. Based on the chromatograms from UPLC system, the peak height ratio between the typical impurities (supposed to be amino acids from hydrolyzed proteins) and GlcN-FMOC-Su were visually compared. The identified condition was also used on insect soluble protein fractions to analyze GlcN.

3.2.4 Hydrolysis of chitin from de-proteined insect fractions

Chitin from insect was hydrolyzed into GlcN prior UPLF-FLR and spectrophotometric analysis. The de-proteined insect flours from both insects were applied for this study. Each sample was dissolved and hydrolyzed in 6M HCl solution (10 mg /3 ml w/v). To get the maximum yield of GlcN, as well as a satisfied recovery of GlcN after extraction and hydrolysis, the effect of heating temperature and incubation time were studied. The mixture solution containing insect sample and 6M HCl was incubated under 110 and 100°C in a heating block for 24 hrs. At the end of each 2-hour interval, a sample was taken and analyzed for GlcN using UPLC-FLR method (See 3.2.6.1). The identified condition was also used on the soluble protein fraction.

3.2.5 Preparing of soluble protein fraction

The soluble protein fractions from dried insect flour were extracted using 0.1M NaCl, pH 10 (prepared by Terhi Lukkari according to the method developed in her master thesis, 2018). For mealworm, the fat was removed by solvent extraction using heptane which was later evaporated. Protein in the defatted mealworm fraction was extracted with 0.1M NaCl where pH was adjusted to 10 using NaOH. The mixture was shaken overnight in cold room and centrifuged in the next morning. After centrifuging, the supernatant was collected and the final pH was adjusted back to 7. Supernatant was freeze dried and stored at -20 °C before use. The cricket flour was directly treated with 0.1M NaCl for protein extraction without de-fatting.

3.2.6 UPLC-FLR analysis of GlcN-HCl

GlcN from hydrolyzed chitin was derivatized with FMOC-Su at measured condition and then analyzed in UPLC-FLR system. The UPLC-FLR method was based on the AOAC Official Method 2005.01 Glucosamine in Raw Materials and Dietary Supplement Containing Glucosamine Sulfate and/or Glucosamine Hydrochloride (Zhou et al. 2005). This method was optimized and validated for GlcN analysis in insect materials.

3.2.6.1 UPLC system

A Waters Acquity UPLC system (Waters Inc.; Milford, MA, USA) with a FLR detector was used for GlcN-HCl analysis. The system consisted of a binary solvent manager, an auto-sampler maintained at 6 °C and a column manager operated at 30 °C. The solvent flow was a gradient mixture of Milli-Q water containing 0.05% trifluoroacetic acid (TFA) (A) and acetonitrile (B): 0~8 min (73:27), 8~9.5 min (73:27 to 0:100), 9.5~10 min (0:100 to 73:27) and 10~14 min (73:27) (Table 1). The chromatogram was recorded at excitation 260 nm and emission 330 nm. The sample injection volume was 2~15 uL which was operated in a partial loop mode. In each sample set, a new calibration curve was obtained by injecting GlcN-HCl standards (0.100~3.486 ng/inj.); meanwhile a control sample (GlcN-HCl standard prepared another time) was run to test the performance of the calibration curve. Chromatographic data was collected and processed using the Waters Empower 2 software. Details of UPLC-FLR method is shown as Appendix 1.

Table 1. Gradient program

Time (min)	Flow (ml/min)	% A	% B
0	0.5	73	27
8	0.5	73	27
9.5	0.5	0	100
10	0.5	73	27
14	0.5	73	27

3.2.6.2 Optimization and validation of UPLC-FLR

GlcN-HCl supplement was used to optimize the UPLC-FLR method based on Zhou et al. (2005). Two reversed-phase C18 columns, high-strength silica T3 (Waters, ACQUITY UPLC ®, HSS, 1.8µm particles, 2.1 mm ID × 150 mm) and ethylene bridged hybrid (Waters, BEH 1.7µm particles, 2.1 mm ID × 100 mm) were evaluated for the separation of GlcN-FMOC from the sample solution. The resolution (calculated according to the formula in the US pharmacopeia; USP) of 2 GlcN-FMOC peaks caused by GlcN isomers in aqueous

solution, the number of theoretical plates (USP) and the peak height for each column were compared. For the selected column, the effect of mobile phase composition on the separation and sharpness of two target peaks was also studied. Different acids including TFA at a serial concentration (0.1%, 0.02%, and 0.05%) and HCOOH (0.5%) were prepared and each acid solution was applied together with ACN as mobile phases to elute out the target compounds, two isomers of GlcN. The shape and resolution of the two GlcN peaks were compared under mobile phases prepared from different acids. For the selected acid, the mobile phase gradient, flow rate (0.32~0.50 mL/min), and column temperature (25, 30 and 40 °C), were tested to optimize the separation condition of two target peaks.

A calibration curve using GlcN-HCl reference standard was constructed and run in each sample set; each standard solution was injected twice. The linearity of the calibration curve, limit of detection (LOD: signal-to-noise ratio, S/N = 3), and limit of quantitation (LOQ: 3 times of the LOD) of GlcN-HCl was studied. For linearity, the determination coefficient was used to evaluate the precision of the calibration curve (of each calibration curve should be ≥0.999). Another GlcN-HCl standard **(**from the same reference standard but weighed another time) was prepared as a control sample to check the accuracy and precision of the calibration. The control sample was injected and analyzed daily with the new calibration curve. The RSD of the experimental values of GlcN-HCl in control sample obtained in at least 3 days with the independent calibration curves was used to evaluate the accuracy and precision of the calibration (the values should be ≤3%); the difference between the average experimental value and true value of the control sample should be within 3%. Data from the control sample was also used to provide information about the stability of GlcN-FMOC derivative.

The accuracy of the analysis method was estimated by recovery test. A sample matrix (10 mg of freeze-dried de-proteined cricket flour) was spiked at one level of a known amount of GlcN-HCl (4.0 mg), in triplicate. The spiked hydrolysis buffer was also tested for recovery. The recovery of each GlcN-HCl was calculated from the measured GlcN-HCl concentration in the spiked samples to the concentration of added GlcN-HCl in the samples.

3.2.6.3 Peak purity and ID confirmation

The purity of each peak shown on the chromatograms in UPLC-FLR was checked using a photo diode array (PDA) detector (PDA; 210~400 nm). The detection was performed at 265 nm and the absorption spectra were recorded by the PDA. The UV spectra from different spots of the same peak were collected and compared. Also the spectra and retention times of peaks between the standard and unknowns to ensure the ID match. Meanwhile the spectra from 2 GlcN-HCl peaks from the same chromatogram were compared, as well as the peaks from different sample solutions.

3.2.6.4 Effect of time on GlcN-FMOC yield under derivatization

GlcN was subject to react with FMOC-Su to yield fluorescence that could be detected by FLR. The optimum derivatization condition was established. GlcN-HCl standard was used to optimize the derivatization of GlcN to get the maximum yield of GlcN-FMOC-Su. A mixture containing 100 µL GlcN-HCl standard solution (2.4 mg/ml in water, pH 11.4 adjusted by adding TEA), 500 µL FMOC-Su solution (30mM in acetonitrile) and 300 µL water was subjected to sonication in water bath under room temperature according to various times (15, 30, 45, 60, 75, 90 min). At the end of each 15-min interval, a sample was taken and analyzed for GlcN-FMOC using UPLC-FLR method (see 3.2.6.1). The identified condition was used on both the calibration standard and the insect materials. Prior to derivatization, the pH of the hydrolysate from insect materials was adjusted to pH 11.4 by NaOH solutions rather than TEA used for GlcN-HCl standard material. After derivatization, the mixture was diluted with mobile phases A/B (1/1, v/v) and mixed well. Each dilution was filtered through 0.2 µm filter (Acrodisc GHP) into an LC vial for UPLC-FLR analysis.

3.2.6.5 Calculation

The amount of GlcN-HCl in samples were calculated using an external standard curve. GlcN-HCl standard was used to construct a calibration plot according to the concentration and corresponding peak area. The calibration curve consisted of 6 different concentration levels which was achieved by varying injection volumes of standard stock solutions

(Appendix 2). There were 2 target peaks shown in each chromatogram, caused by natural GlcN stereoisomers (α and β). The sum of the areas of the peaks was used for the quantification of the GlcN. GlcN-HCl value was converted to GlcNAc by a factor 1.02588. Chitin amount was expressed as GlcNAc as percentage (%) in dry matter.

3.2.7 Spectrophotometric determination of GlcN

The amount of GlcN-HCl in insect samples was also measured using a spectrophotometric method (Tsuji et al. 1969). This method was based on the findings that 2,5-anhydrohexoses, produced by nitrious deamination of glucosamine, react with MBTH to exhibite an intense blue color by addition of a ferric chloride solution. The intense of this color was measured as absorbance at wavelength 650 nm (at the maximum absorbance frequency Amax) using a spectrophotometer. The intensity was proportional to GlcN-HCl concentration. In this case, the greater the absorbance, the higher the GlcN-HCl concentration. The amount of GlcN-HCl in insect samples was calculated using a standard curve. The standard curve was constructed according to the measured absorbance and amounts of GlcN-HCl. The preparation of insect samples and standard solutions and calculation are presented in Appendix 3. The results were calculated and expressed as GlcNAc (g per 100g) in dry matter.

This method was based on the determination of GlcN, thus the hydrolysis of chitin was also required. The preparation of insect hydrolysate was the same as the UPLC-FLR method (see 3.2.5). The hydrolysate of insect material was neutralized by NaOH and the pH was finally adjusted to 6.0~6.5. The neutralized solution was diluted to 10ml using Milli-Q water. Then to 1ml of the mixture, 1ml of 5% $NaNO_2$ and 1ml of $KHSO_4$ were added to produce nitrious acid and the mixture was left standing with occasionally shaking for 15min to make the deamination of GlcN. To remove excess nitrous acid, 1ml of 12.5% $NH_4SO_3NH_2$ was added and the mixture was repeatedly shaken for 5min. Followed was the addition of 1ml of 0.5% MBTH and the mixture was allowed to stand for 60 min. After this, 1ml of 0.5% $FeCl_3$ was added to yield a blue color where the absorbance was read at 650nm against the reagent blank. The readings were used to calculate the GlcN amount.

The recovery test was also performed to evaluate the analysis accuracy of this spectrophotometric method. The tested level was the same as the recovery test in UPLC-FLR method. The sample matrix (10 mg of freeze-dried de-proteined cricket flour) was spiked at one level of a known amount of GlcN-HCl (4.0 mg), in duplicate. The spiked hydrolysis buffer (4.0 mg) was also tested for recovery. The recovery of each GlcN-HCl was calculated from the measured GlcN-HCl concentration in the spiked samples to the concentration of added GlcN-HCl in the samples.

4 RESULTS

4.1 Optimization and validation of UPLC method for GlcN analysis

4.1.1 Column selection, flow rate

GlcN-HCl dietary supplement was used for column selection. Figure 6 shows the chromatograms of GlcN-HCl samples in two column systems. Visually HSS column produced sharper and narrower peaks than those obtained from BEH column. The height of GlcN-FMOC α peak (3.5 min) was about 4-fold taller than that in BEH column (2.1 min) for an equal injection volume from the same sample solution. The theoretical plate number was 2-fold larger on HSS column than that from BET column (2907 vs 1469 for GlcN-FMOC α; 3768 vs 1855 for GlcN-FMOC β). The peak resolution on HSS column was better than that on BEH column (2.5 vs 1.7). Based on the conditions mentioned above, column HSS was found to be superior to BEH column for the separation of two GlcN-FMOC peaks caused by the naturally existing GlcN stereoisomers (α and β) in aqueous solutions. Thus, HSS column was selected for the validation and analysis of GlcN from hydrolyzed sample matrices.

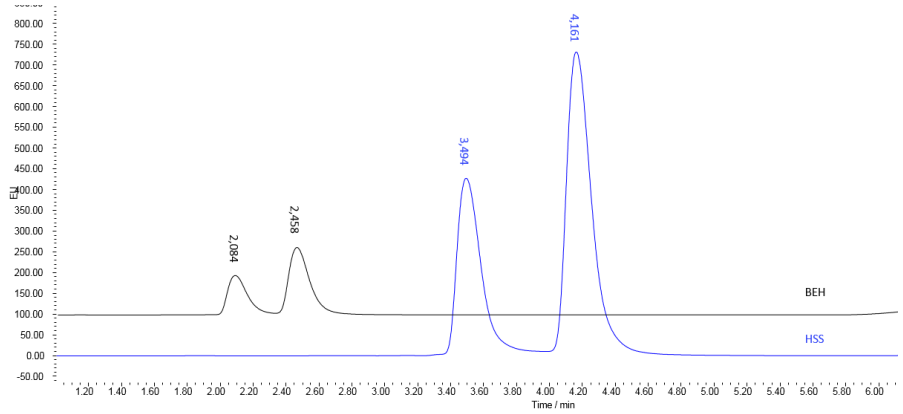


Figure 6. Chromatograms showing the separation characteristics of 2 GlcN-FMOC peaks from HSS and BEH columns, GlcN-HCl dietary supplement solution (0.12 ng/µl), the injection volume, flow rate, column temperature and mobile phase were identical in Session 3.2.6.1.

The effect of mobile phase composition on the shape and separation of two target peaks from HSS column was compared. Mobile phases prepared from both acids, TFA (0.1%, 0.02%, and 0.05%) and HCOOH (0.5%), gave similar sharpness, height and resolution of two GlcN

peaks. Considering the convenience and economic beneficial, 0.05% TFA was selected as the final substance to prepare mobile phase. The effect of ratio between 0.05% TFA and ACN was also studied. The percentage of 0.05% TFA varied from 65% to 80%, and 70% of 0.05% TFA gave a satisfied separation between two target peaks without compromising to the shape of two target peaks. HSS column combined with mobile phases consist of 0.05% TFA and ACN was also tested for a serial flow rate (0.32~0.50 mL/min) and column temperature (25, 30 and 40 °C) with satisfied baseline separation of two peaks within this range, where 40 °C and 0.5 mL/min was selected due to a fast speed.

4.1.2 Validation of UPLC-FLR

The recovery results of added GlcN-HCl after acid hydrolysis by UPLC and spectrophotometric methods are presented in Table 2. The mean recovery of GlcN-HCl added to de-proteined cricket after hydrolysis at one level (4 mg/10 mg, 100%) under 100 °C was higher than that from 110°C (76% vs 61%). A similar GlcN-HCl recovery was obtained for the spiked hydrolysis buffer. The recovery of GlcN-HCl spiked to de-proteined cricket matrix and hydrolysis buffer following spectrophotometric determination were both over 90% under 100°C. The validated UPLC-FLR method enabled a detection limit of 0.00095 ng/inj. (2 µL) and a quantification limit of 0.0033 ng/inj. (7 µL). The linearity of the calibration curve was excellent between 0.0033~24.0 ng/inj. ($R^2 > 0.999$).

Table 2.

Recovery of added GlcN-HCl by UPLC-FLR and spectrophotometric methods after acid hydrolysis of chitin with 6M HCl for 6 hrs under 110 and 100°C

Sample	Spiked amount (mg)	Recovery (%)		Spiked amount (mg)	Recovery (%)	
	UPLC	110°C	100°C	UV	110°C	100°C
De-proteined cricket	4.2	60.8 (±6.8)	75.8 (±9.3)	3.9	NA	92.2 (±4.0)
Hydroysis buffer	4.2	48.5 (±9.3)	74.6 (±9.1)	4.0	NA	96.5 (±0.8)
	No. of determination=3			No. of determination=2		NA: not analyzed

The control sample (standard weighed another time) was analyzed in each sample set on different days. The results obtained from different days were used to estimate the accuracy and precision of the calibration. The RSD of the experimental values of the control sample obtained in 7 days with the independent calibration curves was around 5% (Table 3). The difference between the average experimental value and true value of the control sample was within 3% (Table 3).

Table 3. Precision and accuracy of the calibration curve in 7 days

		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Weight of GlcN-HCl	(mg)	23.8	23.8	23.8	23.8	23.8	23.8	23.8
Calculated	(mg)	24.1	23.0	23.9	25.7	26.5	22.9	24.7
Average	(mg)	24.4 （±5.5 %, RSD）						
Difference between the average value and true value		2.5 %						

To check the peak purity, the UV spectra of the apex. up- and down-slope of each GlcN-FMOC peak from standards and insect samples were compared by using the diode array data processing software (total 5 points for each peak: 2 on the bottom of 2 sides, 2 on the middle point of up- and down-slope, and 1 on the apex.). 5 spectra from each single GlcN-FMOC peak had excellent match from 210 to 400 nm. Spectra from different peaks also showed good match either from standard or insect samples, again indicating no interference or co-elution on both GlcN-FMOC peaks. One example of the chromatograms is displayed in Figure 7.

4.1.3 Optimum of GlcN derivatization with FMOC-Su

GlcN in standard solution was subjected to a derivatization reaction to produce GlcN-FMOC which was determined in UPLC-FLR system. A mixture containing FMOC-Su and GlcN was controlled under measured condition for derivatization reaction. The effect of time was tested on the yield of GlcN-FMOC. At the end of each 15-min interval, a sample was taken

for GlcN analysis. The results were recorded and are shown in Figure 8. The maximum yield of GlcN-FMOC was obtained at 45 min.

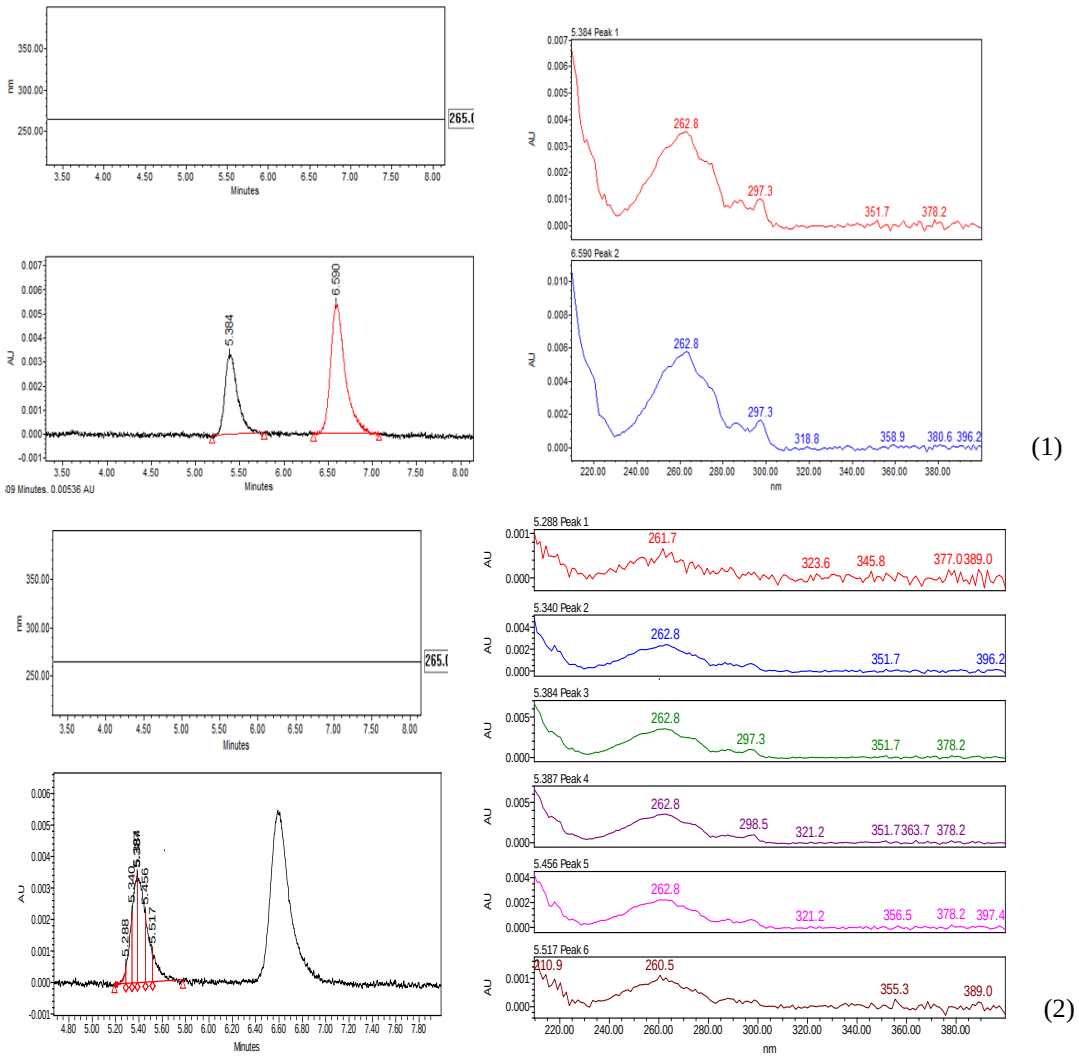


Figure 7. Spectra of two peaks from de-proteined cricket flour in UPLC-FLR system,
(1) comparison of spectra from the two peaks generated by isomers
(2) 5 spectra from one single GlcN-FMOC peak

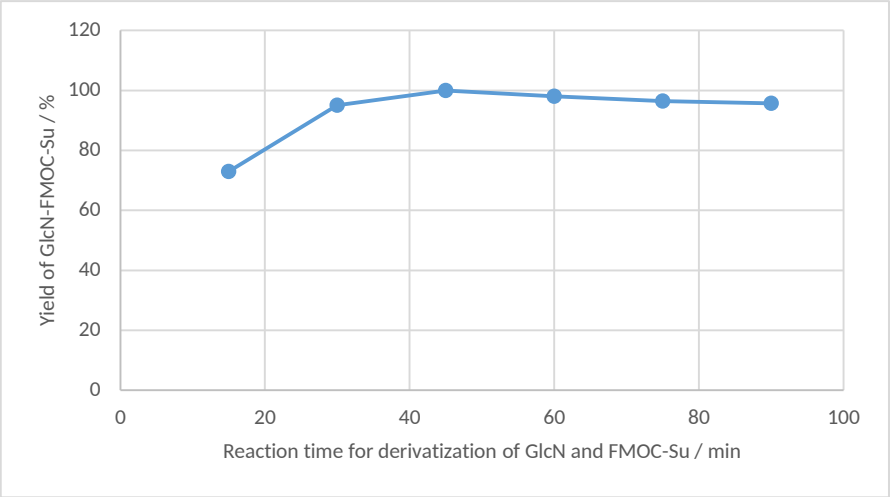


Figure 8. Effect of reaction time of derivatization on GlcN-FMOC yield, sonication in water bath under room temperature

4.2 Major findings on chitin amounts in selected materials

4.2.1 Removal of moisture and protein from dried insect flours

Moisture contents of cricket and mealworm by freeze-drying were and 73.4 ± 0.4% (n = 6) and 74.4 ± 0.5% (n = 5), respectively. Proteins in dried cricket and mealworm were removed by adding 0.5M NaOH solution. The mixture containing dried insect flour and 0.5M NaOH was agitated at room temperature for 0, 2, 4 hrs. For both insects, the impurities were removed to a level without interfering detection of GlcN-FMOC after treated with NaOH for 2 hrs, repeated twice. One example of chromatograms is shown in Figure 9. Two GlcN-FMOC peaks in cricket samples with and without de-proteining were identified by comparing their retention times to those in standard material (chromatograms in blue). Without de-proteining, there were interfering peaks not separated with the target two peaks (chromatogram shown in blank color). There was no interfering compound detected close to the target peaks (chromatogram in green color).

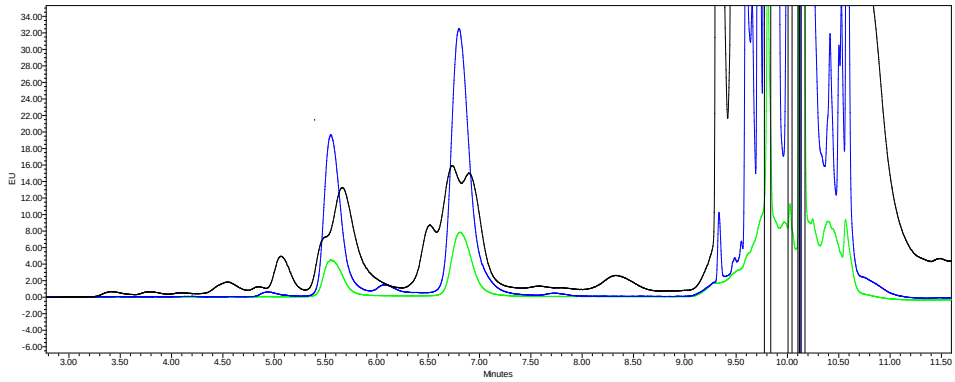


Figure 9. Chromatograms of crickets treated with NaOH (0.5M), with different incubation time
Blue line: GlcN-HCl standard, black line: cricket treated with NaOH (0.5M), incubation time 2h, repeated twice, green line: cricket without de-proteining.

4.2.2 Hydrolysis of de-proteined insect flours

To obtain the degradation product GlcN from chitin, the de-proteined insect flour was hydrolyzed in 6M HCl under 100 °C according to time (2, 4, 6, 7, 8, 10, 12 hrs). At the end of each 2-hour interval, the sample was taken and analyzed for GlcN. The results were recorded and are shown in Figure 10. The maximum yield of GlcN was observed at 6 hrs for both de-proteined cricket and mealworm.

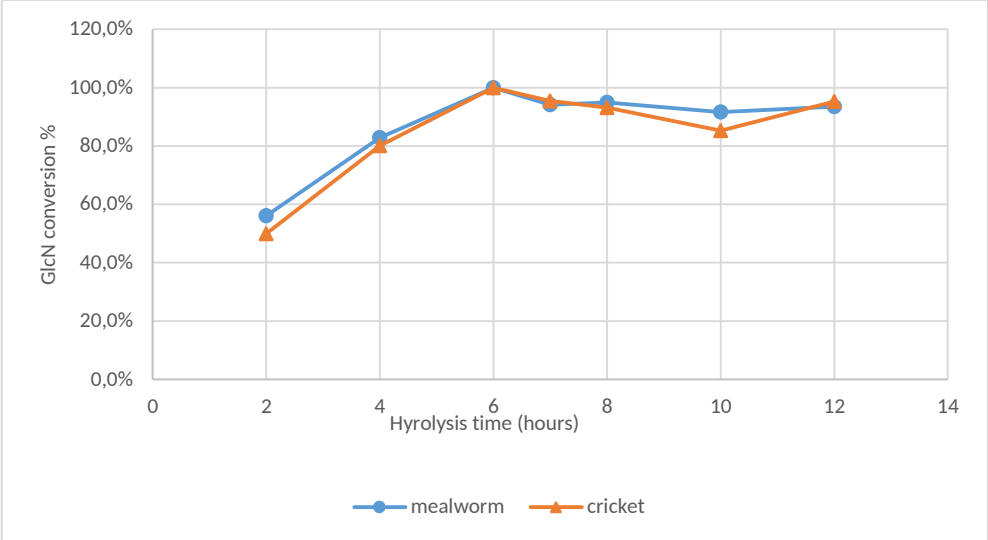


Figure 10. GlcN yield from hydrolyzed chitin in cricket and mealworm (6M HCl, 100 °C)

4.2.3 Chitin amount in selected insects

The major findings on chitin amount expressed as GlcNAc in insect materials were summarized in Table 4. According to UPLC-FLR method, chitin levels were 4.6% and 4.5% D.M. in cricket and mealworm, respectively. There was little chitin found in their soluble fractions, no more than 0.01% in both insects. In all the materials, a slightly higher chitin amount was obtained with the spectrophotometric method than that from UPLC-FLR method. About 5% of GlcNAc was observed in both whole insects and 0.01% in their soluble fractions. Nevertheless, both methods gave identical chitin levels in all the materials, especially in the whole insect body.

Table 4.
Chitin amount in all studied materials by UPLC-FLR and spectrophotometric methods

Sample	UPLC-FLR	UV	UPLC/UV
	%	%	%
cricket	4.6 (± 0.1) (n=5)	4.8 (± 0.3) (n=2)	96
mealworm	4.5 (± 0.0) (n=3)	5.4 (± 0.0)(n=2)	83
Soluble protein of cricket	0.006 (± 0.001)(n=2)	0.01 (± 0.00)(n=2)	60
Soluble protein of mealworm	0.005 (± 0.002)(n=2)	0.01 (± 0.00)(n=2)	50

Values are means ± RSD; dry matter basis, D.M.

5 DISCUSSIONS

5.1 Optimization and validation of UPLC method for GlcN analysis

The calibration curve showed excellent linearity in range 0.0033~24.0 ng/inj. with the determination coefficient more than 0.999 during the study period. The instrumental LOD was much lower than that from HPLC-UV method, AOAO 2005.01 (Zhou et al. 2005), (0.00095 vs 3 ng/inj.), indicating an enhanced sensitivity for UPLC-FLR method. Due to the improved sensitivity of the UPLC-FLR system, it was possible to analyze samples with a GlcN-HCl as low as 0.01 g/100 g, e.g. the soluble protein fractions from selected insects. Data from the control sample indicated good precision, accuracy and stability of the calibration curve. Results of the control sample also showed that GlcN-FMOC derivative once formed was stable without change at 6°C for at least 7 days.

The recovery of GlcN-HCl spiked to the sample matrix (de-proteined cricket flour) following the hydrolysis of chitin was not satisfactory (~75%) using HPLC-FLR method. The reason for this low recovery was unknown. One possible explanation might be an incomplete derivatization of GlcN by FMOC-Su. GlcN from hydrolyzed chitin was subject to FMOC-Su derivatization to introduce fluorescence group detected by FLR. During derivatization, in addition to the target reaction compounds (GlcN and FMOC-Su), water was also added to this mixture (1/5/3 GlcN/FMOC/H_2O, v/v/v) to make the most solubility of FMOC. Without added water, there would be apparently oily droplets in this mixture leading to a low yield of the GlcN-FMOC. However, the added water could decrease the pH of the mixture which might be one reason of incomplete derivatization. In this mixture, the amount of FMOC-Su was always excessive compared to GlcN. FMOC-Su reacts with not only GlcN, but also water. FMOC-Su has a fluorine group with strong fluoresce, as a result, FMOC-Su itself and all its derivatized products including derivatives from water and GlcN would show on the chromatogram after 2 GlcN-FMOC peaks. At the detected level, the excessive FMOC lead to the overload of the FLR detector, which would decrease the life of the FLR. For later study, the work might be focused on optimum of derivatization condition to improve the recovery. Besides, the excess FMOC would also be removed by other organic

solvent e.g. pentane prior UPLC analysis, without compromise to the yield of GlcN-FMOC.

The recovery of GlcN-HCl spiked to cricket sample matrix in spectrophotometric method was over 90%, indicating a better accuracy of this method than that in UPLC-FLR method. In this study, chitin amounts from all insect material using spectrophotometric method was higher than those from UPLC-FLR method, which could be explained by the satisfied GlcN-HCl recovery added to insect matrix. The data also indicated that chitin amount from spectrophotometric method might be closer to the true chitin content in all materials.

Both methods used in this study resulted in similar chitin amounts in cricket and mealworm. UPLC-FLR method was specific for GlcN and the system was able to separate two GlcN-FMOC caused by GlcN isomers (α and β). However, the analysis recovery was not satisfactory indicating partial loss of GlcN during steps prior UPLC-FLR detection. Even though spectrophotometric detection of GlcN gave better analysis recovery than UPLC-FLR, it was not specific to GlcN. Other hexosamines and aldehydes based on the total carbohydrate present in the reaction mixture, regardless of the source, could be de-aminated and then react with MBTH to exhibite a blue color under measuring condition (Sawicki et al. 1961).

The background of both methods to quantify chitin was based on that chitin from insect can be fully degraded into GlcN and then measured using UPLC-FLR or spectrophotometer. The GlcN amount could be transferred into GlcNAc as chitin amount. However, the amount of GlcNAc could be a slight overestimation of chitin. Fully N-acetylated chitin (as a homo-polymer) is rarely found in nature. The fraction of N-acetylated (Fa) units in insect chitin was unknown. Previous studies showed Fa was nearly 1 (between 0.9 and 1.0) in naturally existing chitin (Roberts 1992). Besides, the length of chitin polymer, or degree of the polymerization, also has slight effect on the conversation factor between GlcNAc and chitin. The difference between GlcNAc and chitin caused by Fa and the length would be small, thus, GlcNAc values could be used to roughly estimate chitin levels in insects.

5.2 Major findings on chitin amount in insect materials

In this study, the amounts of chitin in two selected species were around 5% (D.M.) which were much higher than those reported in previous studies. Finke et al. (2007) found that chitin in adult cricket and mealworm larvae were 67.6 and 137.2 mg/kg (D.M.) (0.7% and 1.4% D.M., respectively), indirectly calculated from ADF corrected by subtracting their amino acid contents. ADF method is based on assuming that chitin is the major polysaccharide in insects and it had similar properties of dietary fiber. In ADF method, 0.5M H_2SO_4 is applied to remove acid-labile compounds including carbohydrates and proteins that are not complexed in Maillard products (heat damaged), and fats when they are no more than 5% in dry matter, leaving a fibrous residue. The residue is primarily made up of cellulose and lignin when the sample materials are plant-derived, and insoluble protein complexes from animal-derived materials and heat damaged feeds (AOAC 1990). Chitin is the major polysaccharide in insects and it is not degraded by 0.5M H_2SO_4, which is the reason ADF can be used to estimate chitin amount in insects. In addition, ADF is corrected by subtraction of amino acid content, but amino acid content is not equal to protein amount in ADF fraction, which also adds to the inaccuracy of the final results of chitin. Besides, ADF is not specific to chitin but all non-acid-labile compounds, while UPLC-FLR method applied in this study is specific to chitin due to the detection of GlcN that is the degraded product of chitin polymer.

Another possible explanation for the difference of chitin results between this study and the previous findings might be that chitin amount is highly dependent on the species variations; even within the same species, the growth condition also has significant effect on their chitin content and nutrient levels. In this study, there was slightly more chitin found in mealworm than cricket which was in line with the previous studies. GlcN in soluble protein fractions from cricket and mealworm accounted for quite a small percentage, indicating that 0.1M NaCl was effective to extract protein from insect matrix with removal of most chitin.

The presence of chitin-N resulted in an overestimation of insect crude protein content in cricket and mealworm. In this study, crude protein (Kjehldal method, N x 6.25) in selected insects was around 60% D.M. (Lukkari 2018), with chitin-N accounting for 3% D.M. which was considered a low percentage. All the data supports the fact that chitin-N represents a fairly small fraction of insect's total nitrogen in cricket and mealworm, indicating that N x 6.25 provides a reasonable estimation of crude protein in both insects. This finding is consistent with that previously reported (Finke et al. 2007). Chitin exists only in insect's exo-cuticle and endo-cuticle. However, in most insects, protein, rather than chitin, is the predominant compound in the cuticle (Kramer et al. 1995).

6 CONCLUSIONS

Two methods, UPLC-FLR and spectrophotometric methods, were applied to determine GlcN from hydrolyzed chitin in insect materials and both analysis resulted in identical chitin amounts. UPLC-FLR method displayed good performance on calibration and showed high specificity on GlcN determination. However, the recovery of added GlcN-HCl into insect matrix following hydrolysis was not better than that from spectrophotometric method. Further studies to improve recovery of GlcN-HCl using UPLC method is to be conducted. This work also suggested that removal of protein prior to hydrolysis of chitin was essential for both UPLC-FLR and spectrophotometric analysis of GlcN. Alkaline treatment (0.5M NaOH) was proved to be effective for de-protein of selected insects.

Chitin levels in cricket and mealworm were similar (~5% D.M.) where chitin was expressed as the amount of GlcNAc. This resulted in a slight over estimation of chitin levels in insect materials due to the fraction of N-acetylated (Fa) units and the length of chitin in insects. Further studies to analyze the Fa and polymerization degree of insect chitin should be conducted in the coming years, and the results can be used to correct chitin values from HPLC-FLR and spectrophotometric methods. Chitin-N accounted for a small percentage of total nitrogen, indicating that 6.25 × N could be used to estimate true crude protein content in cricket and mealworm.

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Appendices

Appendix 1. UPLC-FLR conditions for GlcN determination

Instrument

Waters ACQUITYTM Ultra Performance LC (UPLC®) (binary pump-system, auto-sampler, FLR-detector, and PDA-detector).

Stationary phase/column

Waters, ACQUITY UPLC ®, HSS T3, 1.8µm silica particles, 2.1 mm ID × 150 mm
(HSS T3, 2.1 × 150 mm, 1.8µm)

Mobile phases

Mobile phase A: water containing 0.05% TFA, pH 2.4:

0.5 ml TFA was added to 1L volumetric flask containing around 900 ml water, and then the solution was diluted to volume with Milli-Q water and mixed well. The pH was confirmed with pH meter. The solution was filtered with 0.2 µm membrane before use.

Mobile phase B: acetonitrile

Run conditions

Column temperature: 30°C
Cooler temperature: 6°C
Flow rate: 0.5 ml/min
Elution: gradient, according to Table 1.
Injection volume: 5µl
FLR: excitation 260 nm; emission 330 nm (λex=260 nm λem=330 nm)

Scheme of the chromatographic run:

Table1. Gradient program

Time (min)	Flow (ml/min)	% A	% B
0	0.5	73	27
8	0.5	73	27
9.5	0.5	0	100
10	0.5	73	27
14	0.5	73	27

Appendix 2. Calibration curve for UPLC-FLR determination of GlcN 1/2

A standard curve was prepared for each UPLC sample set separately. Each standard point was injected into the UPLC system twice at least.

Standard working solutions

- **Stock solution 1**
  The standard stock solution was prepared by dissolve GlcN-HCl in water (24 mg/10 ml, pH adjusted to 11.4-11.5 by pipetting 75 µL TEA).
- **FMOC-Su derivatization solution 2**
  30 Mm, 100 ± 1.0 mg FMOC-Su dissolved in 10 Ml acetonitrile
- **Solution 3**
  A mixture containing 1 100 µl and 2 500 µl was pipet into 5 ml volumetric flask. Then the mixture was sonicated in water bath for 45 min under room temperature. The derivatized solution was diluted with mobile phases A/B (1/1, v/v).
- **Std. 1:100**
  Solution 3 was diluted to 1:100
- **Std. 1:1000**
  Solution 3 was diluted to 1:1000

Std.1:100 and Std.1:1000 were used to construct the 6-point calibration curve. Different concentrations of GlcN-HCl was obtained by varying the injection volume (2~10 µl) according to Table 2.

Table 2. GlcN-HCl concentrations for 6-point calibration curve

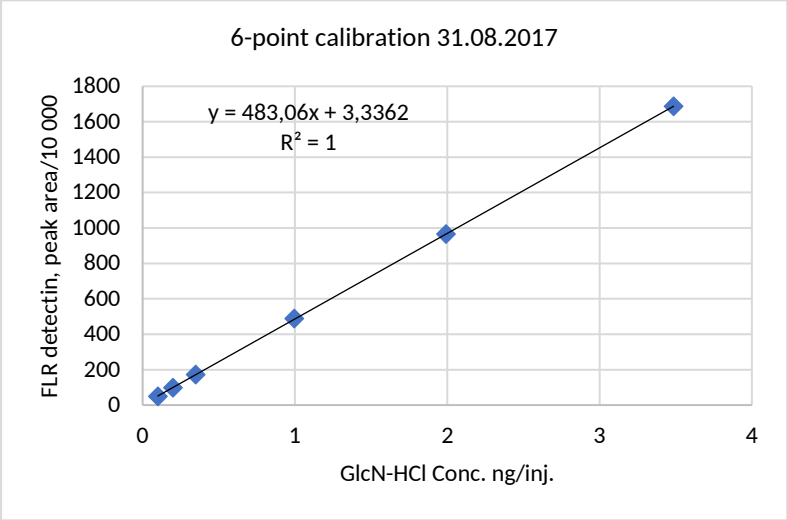
	Concentration (ng/µl)	Injection vulume (µl/inj.)	Amount (ng/inj.)
Std. 1:100	0.48	7	3.36
Std. 1:100	0.48	4	1.92
Std. 1:100	0.48	2	0.96
Std. 1:1000	0.048	7	0.336
Std. 1:1000	0.048	4	0.192
Std. 1:1000	0.048	2	0.096

Appendix 2. Calibration curve for UPLC-FLR determination of GlcN 2/2

Calculation

The mass of the GlcN-HCl at each injection was calculated and used to construct the calibration curve against the peak area of the sum of the two GlcN-HCl peaks. The calibration curve was done using linear regression and the R^2 was given. The calibration curve was used to quantify GlcN-HCl hydrolysed from insect chitin. The amount of GlcN-HCl was finally transformed into GlcNAc.

Example of a calibration curve:



Equation of the standard curve: Y = a x + b

The GlcNAc amount was calculated according to the following equation:

$$\%\,\mathbf{g/g} = (\mathbf{P} - \mathbf{b}) \times \mathbf{100} \times \mathbf{1.02588} / (\mathbf{a} \times \mathbf{D} \times \mathbf{W})$$

P = peak area, the sum of two GlcN-FMOC peaks
a=slope of the calibration curve
b= intercept of the calibration curve
D = the dilution factor
W = the amount of insect sample weighed, mg.
1.025877661 = 221.21/215.63, conversion factor from GlcN-HCl to GlcNAc

Appendix 3. Spectrophotometric determination of GlcN-HCl 1/3

The amount of GlcN-HCl in insect samples was calculated using a standard curve which was constructed according to the measured absorbance and amounts of GlcN-HCl in standard.

GlcN-HCl standard solutions

- **Stock solution I**
  30 mg/ 10 ml, (3 mg/ml, 3000 μg/ml)
- **Std. 1:10 II:**
  1ml of stock solution was pippet into 10 ml (300 μg/ml)

Standard solutions with different concentrations (containing GlcN-HCl 0.3~30μg/ml) were obtained by pipetting various amount of Std. 1:10 II into 10 ml volumetric flasks according to Table 3. Each standard was prepared in duplicate, the standard curve was analyzed before the real insect samples.

Table 3. Preparation of standard solutions with different concentrations

	Conc. GlcN-HCl (μg/ml)	Pippetted volume (μl)
1	0.3	10
2	0.99	33
3	6	200
4	12	400
5	18	600
6	24	800
7	30	1000

Procedures of measuring Std.

- To 1 ml of each standard solution, 1 ml of 5% $NaNO_2$ and 1 ml OF 5% $KHSO_4$ were added. The mixture was then left standing with occasionally shaking for 15 min, upon which the deamination was completed.
- Then 1 ml of 12.5% $NH_4SO_3NH_2$ was added and the mixture was repeatedly shaken for 5 min. Excess nitrous acid was removed during this process.
- 1 ml of 0.5% MBTH was added and the mixture was allowed to stand for 60 min
- Finally, 1 ml of 0.5% $FeCl_3$ was added and reacted for at least 30 min.
- The absorbance was read at 650 nm against the reagent blank.

Sample preparation

Deproteined insect flour was hydrolyzed using 6 M HCl (10 mg /3 ml w/v). 3 ml of the hydrolysate was neutralized by adding 1.4 ml 12 M NaOH, the pH was finally adjusted to 6.0~6.5 by adding NaOH solutions.

The mixture was transferred into a 10-ml volumetric flask which was filled by Milli-Q water.

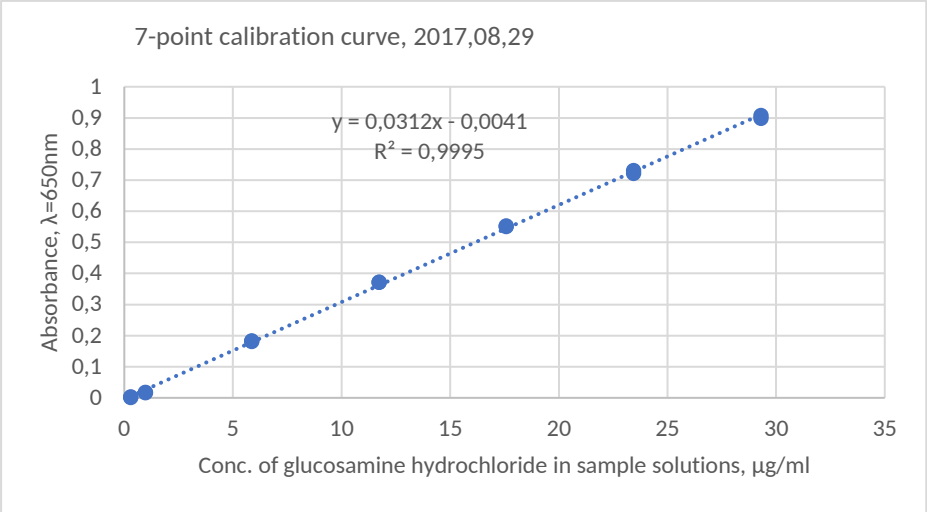
Into the test tube 1 ml of the neutralized solution was pippeted and the following procedures was the same as the standard solutions. The solution was diluted in proper ratio to let the absorbance fell into the linear range.

Appendix 3. Spectrophotometric determination of GlcN-HCl 2/3

Calculation

The standard curve was constructed according to the measured absorbances and the concentrations/amounts given in the table,
Y-axis, standard absorbances after background deduction
X-axis, concentrations of GlcN-HCl (µg/ml)

Example of a standard curve



Equation of the standard curve: Y = a x + b

The amount of GlcNAc was calculated according to the following equation:

$$\% \text{ g/g} = (A_{sample} - \text{A}_{blank} - \text{b}) \times 100 \times 1.02588/(\text{a} \times \text{D} \times \text{W})$$

A_{sample}= measured absorbance of a sample
A_{blank}= measured absorbance of a sample-blank
a=slope of the standard curve
B= intercept of the standard curve
D = the dilution factor
W = sample amount (insect weighed, mg).
1.025877661 = 221.21/215.63, conversion factor from GlcN-HCl to GlcNAc

Appendix 3. Spectrophotometric determination of GlcN-HCl 3/3

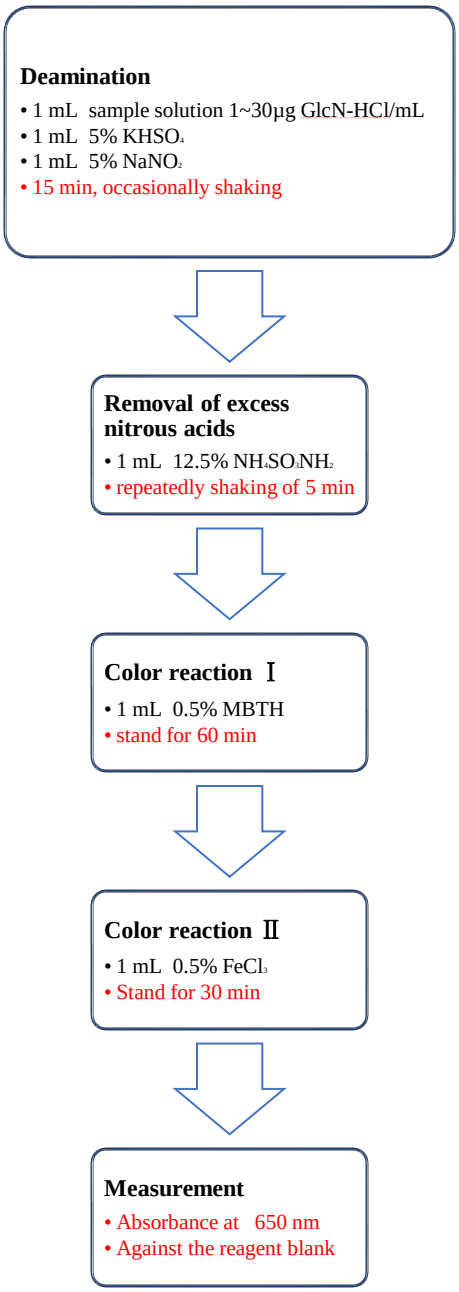


Figure 1. Scheme of GlcN-HCl measurement (Tsuji et al. 1969)

Note: Make duplicate for each sample; measure each solution in duplicate